CHAPTER 7

# Insect Cell Expression Technology

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Significant advances in biology have resulted from the use of insect cell expression systems to facilitate the production of many types of eukaryotic and prokaryotic proteins and as a tool to study the regulation of gene expression. The recent development of rapid and efficient insect cell expression systems—particularly transient systems based on baculovirus vectors, and those based on stably transformed insect cell lines—have contributed to the widespread use of this technology as an acceptable alternative to many of the well-established bacterial and mammalian cell-based expression systems. The major features of each expression system are compared, and novel applications of insect cell expression technology are briefly described.

## 7.1 INTRODUCTION

The development of expression systems which permit the production of proteins in heterologous hosts is critical for the commercialization of products developed through the application of modern biotechnology. Discoveries brought about by the isolation and characterization of genes at a molecular level have been important for accelerating the development of genetics, biochemistry, and cellular biology, which have resulted in significant advances in the diagnosis and treatment of diseases and the development of improved methods for the production of food and other agricultural products.

Prokaryotic expression systems are faster and cheaper to use than most eukaryotic

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expression systems. Plasmid and bacteriophage vectors are generally small and easily manipulated, and proteins encoded by heterologous genes inserted into these vectors are often expressed at high levels (1–4). Well-understood engineering principles can be used to optimize the conditions for growth and induction in large-scale fermenters (5,6). One major problem that is often encountered with expression of cloned genes at high levels in bacterial systems, however, is the misfolding and accumulation of polypeptides in inclusion bodies (7–10). While separation of inclusion bodies often provides an excellent and inexpensive means of purification for commercial proteins, sophisticated solubilization and renaturation procedures are often required to produce material which is biologically active, and these methods may be inefficient or ineffective for all but the smallest proteins. A second major problem is that bacteria lack many of the pathways found in eukaryotic cells that are responsible for chemical modification or site-specific proteolytic cleavage of proteins (11,12). Glycosylation, for example, can be important in directing the targeting and assembly of a protein and can modulate its biological activity by influencing its heterogeneity, immunogenicity, conformational stability, and *in vivo* clearance rate (13–17).

Because of these problems, a wide variety of systems have been developed to express proteins in eukaryotic hosts (18–21). Expression systems based on vectors which propagate in yeast (22–27), filamentous fungi (28), mammalian cells (18,21,29–33), or insect cells (34–37) have been most widely used. In the following sections, we will briefly describe (a) recent advances in the development of insect cell expression vectors and (b) novel applications of this technology, particularly for genes that cannot be efficiently expressed in other systems.

## 7.2 EUKARYOTIC EXPRESSION SYSTEMS

Eukaryotic expression systems can be divided into two types: those that involve transient or stable expression of heterologous genes from transfected DNA molecules, and those that involve helper-independent viral expression vectors (18,20, 21,29). Vectors used for stable expression contain a complete eukaryotic transcriptional unit inserted into a bacterial replicon. A selectable marker may be included in the vector, or it may be on a separate replicon and co-transfected into the cell. The DNA integrates at a low frequency into the host genome and usually directs the expression of the desired protein at low levels. In most cases, these systems are used to direct the constitutive expression of nontoxic proteins that are secreted into the medium.

A variety of helper-independent eukaryotic expression vectors have been developed, which are based on large double-stranded DNA viruses (reviewed in reference 29). These include poxviruses such as vaccinia virus, herpesviruses, adenoviruses, and baculoviruses. Manipulation of the genome of these large DNA viruses is usually accomplished by homologous recombination between a cloned segment of viral DNA and genomic viral DNA in cultured cells. Both can be introduced into uninfected host cells by co-transfection or by transfection of the transfer vector into previously infected cells. These viruses are usually lytic in cultured cells and are often used for small-scale transient expression of heterologous genes in specific cell types. The most versatile mammalian system is based on a vaccinia virus that has been modified to contain genetic elements from bacteriophage T7 and the *E. coli lac* operon (38.39).

The selection of vectors which permit the expression of heterologous genes in insect cells is limited compared to what is available for use in mammalian cells. The major reason is the vast difference in resources allocated to fundamental studies of mammalian cells, primarily directed at health care, compared to studies of insect cells, funded primarily by agencies interested in agricultural pest control. One reason for the limited selection of insect cell expression vectors has been the lack of promoters and selectable markers which have been characterized and shown to work well in a variety of insect cell lines. Although there are a variety of promiscuous promoters that work well in many mammalian cell lines, no systematic studies have been carried out, so far, to determine if any of them function in insect cells.

Concerns about possible differences in the nature of post-translational modifications carried out by insect cells compared to mammalian cells and their role in determining the biological activities of a protein have also contributed to the reluctance of some researchers to use insect cell expression systems. Fortunately, these fears are often unfounded, because literature describing the use of insect cell expression systems, particularly baculovirus vectors, point to hundreds of successes and few failures for proteins that are inefficiently expressed by mammalian or bacterial expression systems (see below). The major features of different insect cell expression systems and their applications are compared in Table 7.1 and discussed in the following sections.

## 7.3 STABLY TRANSFORMED INSECT CELL LINES

Although more than 400 insect cell lines have been established in the last 30 years (40–43), very few have been developed into systems which permit the selection of stably transformed cell lines. Cell lines derived from dipteran insects, including the mosquito (*Aedes albopictus*) and the fruitfly (*Drosophila melanogaster*), and lepidopteran insects, such as the fall armyworm (*Spodoptera frugiperda*), have been most commonly used.

The development of stably transformed mosquito cell lines was important for fundamental studies directed at the control of mosquito-borne disease. Early progress in this area was limited until studies demonstrated that polybrene is superior to calcium phosphate for the transfection of plasmids into cultured mosquito cells (44,45). These studies demonstrated that transfected plasmids were maintained in the cells for at least 4 days and that heat treatment could induce transient expression of chloramphenicol acetyltransferase under the control of the *Drosophila* heat shock protein (*hsp*) 70 promoter. New vectors containing genes that function as dominant selectable markers (such as dihydrofolate reductase) and the use of strong host promoters (from *Aedes* ribosomal proteins L8 and L31) should accelerate the development of stably transformed mosquito cell lines (46). Stable incorporation of over 10,000 copies of a vector containing a gene conferring resistance to hygromycin under the control of the *Drosophila hsp* promoter has also been reported (47).

A variety of heterologous genes have been successfully expressed in stably transformed *Drosophila* cell lines (48,49). In this system, a variety of selectable markers were tested, including those that confer resistance to neomycin, methotrexate, or hygromycin. Also tested were a variety of promoters, including the SV40 early promoter, the 5′ long terminal repeat (LTR) of a *Drosophila copia* element, and the *Drosophila* metallothionein promoter. The best results were usually obtained when an expression vector, which uses the *Drosophila* metallothionein promoter to direct

**Table 7.1 Insect Cell Expression Systems**

| Type | Host | Vector | Promoter | Selection | Notable Features | Reference |
|---|---|---|---|---|---|---|
| Stably transformed cells | *Drosophila melanogaster* | Selection and expression plasmids | *Drosophila* metallothionein | Neomycin, Methotrexate, Hygromycin | Rapid selection of clones, inducible | 48, 49 |
| | *Aedes albopictus* | Selection and expression plasmids | *Drosophila* heat shock, *Aedes* L8, and L31 ribosomal proteins | Methotrexate, Hygromycin | | 46, 47 |
| | *Spodptera frugiperda* | Selection and expression plasmids | AcNPV IE1(0) | Neomycin | Efficient secretion and processing of complex proteins, low expression levels | 56 |
| Transient, helper-independent virus vectors | *Spodptera frugiperda, Trichoplusia ni* | AcNPV | AcNPV polyhedrin, *p10*, or basic protein | See text | Abundant expression levels in cultured cells for most heterologous genes | 35, 36 |
| | *Bombyx mori* | BmNPV | BmNPV polyhedrin | See text | Production in large *B. mori* larvae | 60, 98 |

*Note:* Expression vectors based on other insect viruses are discussed in the text.

transcription of the desired heterologous gene, was introduced into *Drosophila* Schneider-2 cells by co-transfection with a selection vector that confers resistance to hygromycin. Stable cell lines could be obtained after 3 weeks of selection, and the number of copies of the integrated transcription cassette could be altered by manipulating the ratio of the two plasmids. Heterologous gene expression was tightly controlled and could be induced more than 50-fold by incubation in medium containing heavy metals such as cadmium. This system has been used to express *E. coli* galactokinase (49), p21 *ras* oncogene product (48), human tissue plasminogen activator (t-PA) (50), HIV gp160 (51), and HIV gp120 (52). Similar approaches have also been used by others to select stably transformed Schneider-2 cells that express human α-1-antitrypsin (53), human t-PA (54), and bovine dopamine β-hydroxylase (55).

Stable transformation of *Spodoptera frugiperda* cells has been performed by using vectors that contain a weak baculovirus promoter derived from the *ie-1* immediate-early gene of the *Autographa californica* nuclear polyhedrosis virus (56). Cells were transformed by transfecting with a mixture two vectors, one containing a gene conferring neomycin resistance under the control of the *ie-1* promoter, and the other containing a reporter gene under the control of the *ie-1* promoter, or a derivative of the delayed early *39K* promoter. Expression of *E. coli* β-galactosidase in stably transformed cells was more than 1000-fold lower than in cells infected with a recombinant baculovirus containing the *E. coli lacZ* gene under the control of the very late polyhedrin promoter. Expression of human t-PA was also very low (~1 μg/$10^6$ cells) in both systems. Expression was continuous and stable, and processing and secretion of t-PA was more efficient in the stably transformed cell lines than in the lytically infected insect cells. This system may be useful for fundamental studies of insect cells and baculoviruses (57), but is unlikely to displace any of the mammalian cell-based expression systems for routine protein production.

## 7.4 BACULOVIRUS EXPRESSION SYSTEMS

Recombinant baculoviruses have become widely used as vectors to express heterologous genes in cultured insect cells and insect larvae. Heterologous genes are usually placed under the control of the polyhedrin or *p10* promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV). The polyhedrin and *p10* genes are not essential for replication or maturation of the virus in cultured cells, and they are highly transcribed during the very late stages of infection. In most cases, the recombinant proteins are processed, modified, and targeted to their appropriate cellular locations, where they have the expected biological properties (34–36, 58–60).

A number of unique features distinguish the baculovirus expression vector system from other expression systems:

1. High levels of heterologous gene expression are often achieved compared to other eukaryotic expression systems, particularly for intracellular proteins. In many cases the recombinant proteins are soluble and easily recovered from infected cells late in infection when host protein synthesis is diminished.
2. The rod-shaped nucleocapsids contain a single molecule of supercoiled double-stranded DNA. There do not appear to be rigid packaging constraints because

the viral genome can expand to accommodate large inserts. Recombinant baculoviruses have a defined genetic structure which can be easily confirmed by DNA hybridization, DNA amplification, or restriction profile analysis.

3. Baculoviruses have a restricted host range, limited to specific invertebrate species. The viruses are safer to work with than mammalian viruses because they are noninfectious to vertebrates and plants. Unlike many vertebrate cell lines, most susceptible insect cell lines are not transformed with pathogenic or infectious viruses and can be cared for under minimal containment conditions. Helper cell lines or helper viruses are not needed because the baculovirus genome contains all the genetic information needed for propagation in a variety of cell lines from different insect species.
4. A variety of cell lines have been developed and characterized which are susceptible to AcNPV and easily propagated in suspension or as monolayers. Prolific cell lines are available which grow well in suspension cultures, permitting the large-scale production of recombinant proteins in bioreactors. Uninfected cells can be maintained in continuous culture in small-scale bioreactors and used as starter cultures for large-scale batch production of cells infected with different stocks of recombinant viruses.
5. Recombinant viruses can be used to infect insect larvae, by injecting the budded virus into the hemocoel or by ingestion of an occluded or preoccluded form of the virus. The recombinant protein can be harvested from the hemocoel if it is secreted from infected cells. Intracellular proteins can be recovered from specific infected tissues or from homogenized whole larvae.
6. Expression of hetero-oligomeric protein complexes can be achieved by simultaneously infecting cells with two or more viruses or by infecting cells with recombinant viruses containing dual- or multiple-promoter expression cassettes.

Recent advances in the development of improved baculovirus vectors and novel applications of this technology are described in the following sections. Other developments, such as isolation of improved cell lines, the optimization of growth in large-scale bioreactors, the biological properties of proteins expressed in insect cells, and other areas that are the subject of earlier detailed reviews, are only briefly described.

## 7.5 THE BIOLOGY OF BACULOVIRUSES

Baculoviruses are a large group of invertebrate DNA viruses characterized by the rod-shaped morphology of the virion. Virions consist of one or more nucleocapsids surrounded by an envelope and contain a circular double-stranded DNA genome ranging in size from 80 to 220 kb in length. Over 500 types of baculoviruses have been characterized which are generally named according to the genus and species of the host where they were first discovered. The majority have been isolated from seven major insect orders (Lepidoptera, Diptera, Hymenoptera, Trichoptera, Coleoptera, Neuroptera, Crustacea), and a few have been isolated from Arachnida and Crustacea. Individual viruses usually have a restricted host range, limited to one or more closely related species, and do not infect vertebrates or plants.

Two major subfamilies of the Baculoviridae family of viruses are recognized (Table 7.2). These include the Eubaculovirinae and the Nudibaculovirinae. Nuclear polyhedrosis viruses (NPV) and granulosis viruses (GV), which are two distinct

**Table 7.2. Baculovirus Taxonomy**

| Subfamily | Genus | Subgenus | Hosts | Structural features | Type species |
|---|---|---|---|---|---|
| Eubaculovirinae (occluded baculoviruses) | Nuclear polyhedrosis viruses | Multiple nucleocapid viruses | Lepidoptera | Multiple nucleocapsids per virion envelope, many virions per polyhedral occlusion body (0.1–15 μm diameter) | *Autographa californica* MNPV |
| | | Single nucleocapsid viruses | Lepidoptera, Diptera, Hymenoptera Trichoptera, Coleoptera, Neuroptera, Crustacea | Single nucleocapsids per virion envelope, many virions per polyhedral occlusion body (0.1- to 15-μm diameter) | *Bombyx mori* SNPV |
| | Granulosis viruses | | Lepidoptera | Single nucleocapsids per virion envelope, single virions per ovicylindrical occlusion body (0.2 × 0.5 μm) | *Trichoplousia ni* GV |
| Nudibaculovirinae (nonoccluded baculoviruses) | Nonoccluded baculoviruses | | Coleoptera, Diptera, Mites, Crustacea | Single nucleocapsids per envelope | *Oryctes rhinoceros* nonoccluded BV |

genera of the Eubaculovirinae, produce virions which are occluded late in infection within a paracrystalline matrix of viral proteins. Nuclear polyhedrosis viruses produce occlusion bodies which are polyhedral in shape and contain many viral particles, while granulosis viruses produce ovicylindrical occlusion bodies which contain only one or rarely two or more particles. Members of the Nudibaculovirinae do not produce occluded virions at any point in their life cycle.

The most widely studied baculovirus is the *Autographa californica* nuclear polyhedrosis virus, which was first isolated from an infected alfalfa looper. Although most baculoviruses have a restricted host range, AcNPV can infect 39 species of moths (61). Continuous cell lines are not available for all of these species, and AcNPV is usually propagated in cell lines derived from the fall armyworm *Spodoptera frugiperda* or from the cabbage looper *Trichoplusia ni*. Although much of the early work on AcNPV was directed toward the development of viral pesticides (reviewed in references 62 and 63), most of the current knowledge about the biology and genetics of this virus is the result of intense efforts to develop improved expression vectors. A dramatic acceleration in this area is expected when the sequence analysis of the AcNPV genome (determined in the laboratory of Dr. R. D. Possee) is published.

The life cycle of AcNPV is complex (Figure 7.1) and serves as a model for the replication of most other baculoviruses (reviewed in references 64–67). Two morphologically distinct, but genetically identical, forms of the virus are produced at different times in infected cells (Figure 7.2). Budded virus particles (BV) are produced early in infection and serve to spread the virus to other tissues within the insect larvae. Occluded or polyhedra-derived viral particles (OV or PDV), produced late in infection, are responsible for the long-term survival of the virus and transmission from insect to insect. The occluded particles (also called *occlusion bodies* or *polyhedra*) contain many nucleocapsids surrounded by a paracrystalline matrix, composed primarily of polyhedrin, a major structural protein (Figure 7.3). This matrix serves to protect the virus from inactivation by desiccation or sunlight. Occluded baculoviruses are fairly stable in the environment, and they can be recovered from treated soils after more than 20 years.

Caterpillars feeding on contaminated plant material ingest occluded viral particles which are solubilized in the alkaline environment of the midgut to release virions that enter adjacent cells by endocytosis (Figure 7.1). The virions are uncoated as they enter the nucleus where viral genes are expressed in a temporally coordinated cascade. Four distinct phases of transcription are recognized and designated as immediate-early, delayed-early, late, and very late. Most genes are expressed during a single phase, but several are known to be under the control of promoters that are active during several phases. Transcription of viral promoters initiate within highly conserved promoter elements: CGT for early genes and TAAG for late genes, which are generally located 30 to 90 bp upstream from an ATG translational start codon (68,69). Some promoters contain both of these elements, implying functional roles for the corresponding gene products during early and late stages of infection. DNA replication begins around 6 hours post-infection (hpi) when synthesis of many early gene products declines and production of many nucleocapsid structural proteins begins (6 to 20 hpi). Budded viruses are released from the plasma membrane between 10 and 24 hpi, but production of occluded virus continues in the nucleus into the very late phase (up to 72 hpi) prior to cell lysis. Different tissues within a larvae support the viral infection to different extents, with most accumulation of virus taking place in the fat body, hypodermis, hemocytes, and tracheal matrix

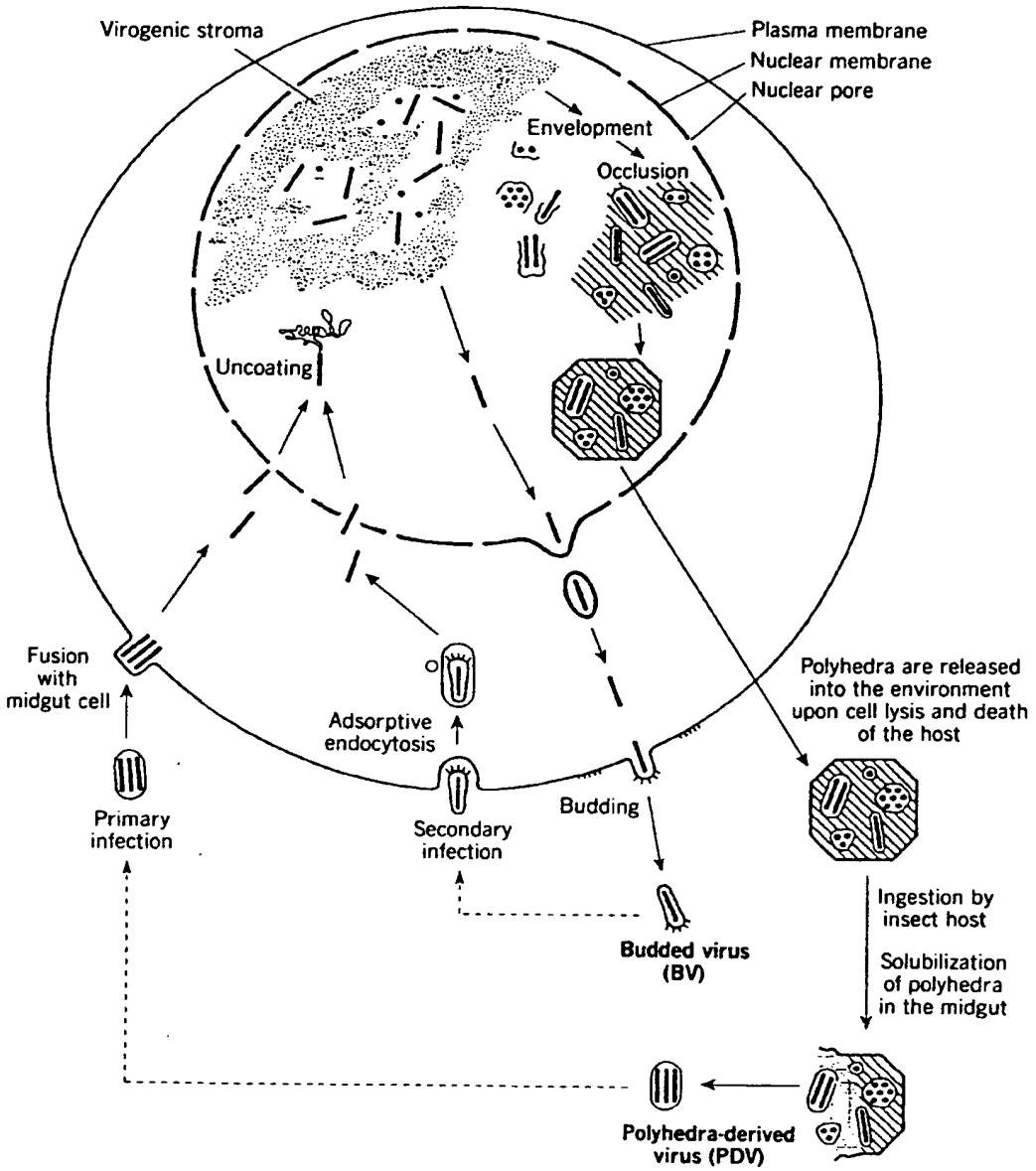


**Figure 7.1.** Nuclear polyhedrosis virus life cycle. Polyhedra dissolve in the alkaline environment of the midgut to release nucleocapsids which are uncoated as they enter the nucleus. Viral genes are expressed a temporally coordinated cascade to control DNA replication and the synthesis of structural proteins. Budded viruses spread the infection to other tissues within the host organism. Occluded viruses, produced late in infection, are released to the environment when infected cells lyse and the host organism dies. [Diagram used with permission of George Rohrmann (64).]

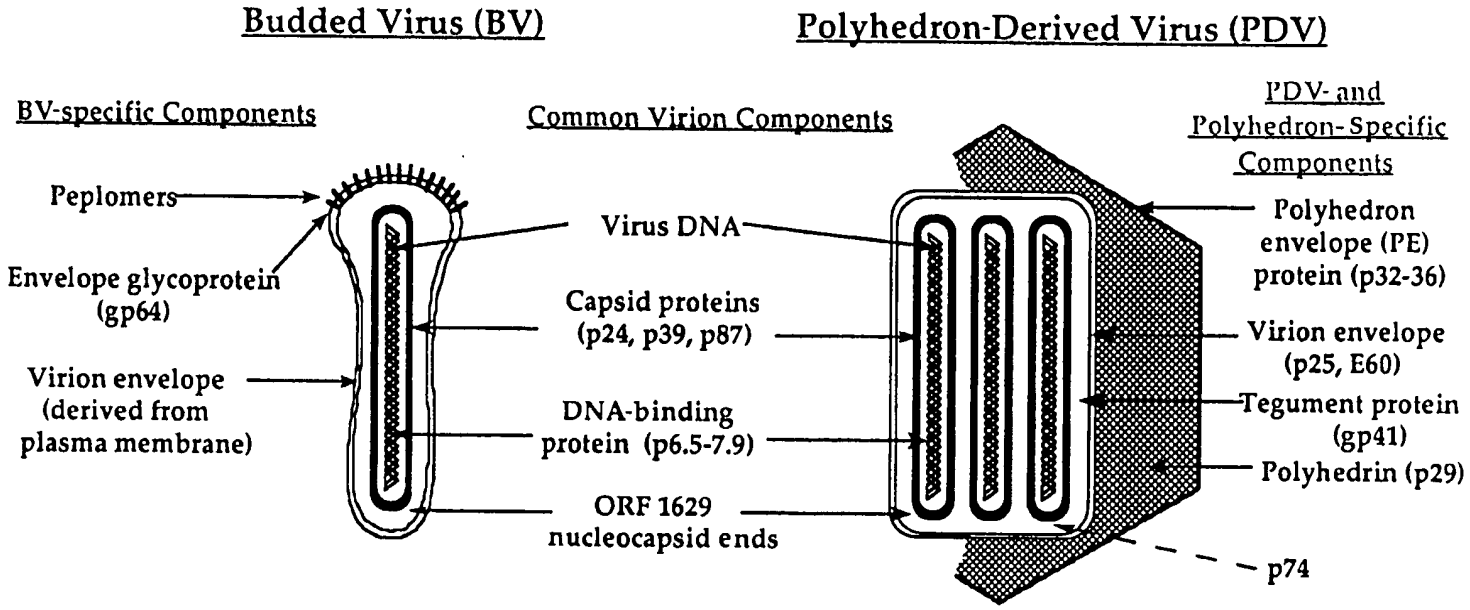


**Figure 7.2.** Schematic diagram of the structural components present in the budded (BV) and polyhedra-derived (PDV) forms of baculoviruses. The location of p74 has not been determined. [Diagram used with permission of George Rohrmann (169).]

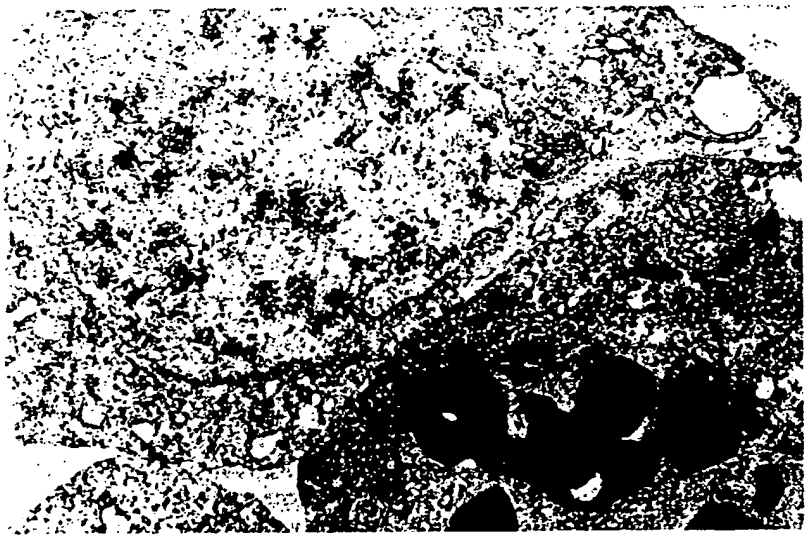

**Figure 7.3.** Immunogold staining of AcNPV-infected fat body cells from *Trichoplusia ni*. One cell is infected with the recombinant virus AcUW2(B).JHE, which expresses juvenile hormone esterase (JHE) under the control of the *p10* promoter (170). An adjacent cell is uninfected. Prominent features are the enveloped nucleocapsids occluded within the polyhedra and fibrillar structures within the infected cell. The section is enhanced for JHE by silver staining which can be seen as black dots in cytoplasm of the infected cell. [Micrograph courtesy of T. F. Booth, B. C. Bonning, and B. D. Hammock.]

(70,71). Although 20 to 30 polyhedra are typically seen within the nucleus of AcNPV-infected *Spodoptera frugiperda* cells late in infection, higher numbers are observed in a few cell lines from other species (72). Late instar larvae may product up to $10^{10}$ polyhedra before death, which account for up to 30% of the dry weight of an infected larvae (73).

## 7.6 CONSTRUCTION OF RECOMBINANT BACULOVIRUSES

Recombinant baculoviruses that contain heterologous genes are usually constructed in two steps. A heterologous gene is first inserted into a baculovirus transfer vector downstream from a viral promoter that is flanked by baculovirus DNA from a nonessential locus, usually the polyhedrin gene or the *p10* gene. The baculovirus transfer vector and genomic viral DNA are then introduced into insect cells where they recombine to produce a virus containing an integrated copy of the heterologous gene. Recombinant viruses are usually identified by an altered plaque morphology and purified through several rounds of plaque assays (Figure 7.4). Detailed protocols, describing many of the steps in this process, are described in several laboratory manuals (35,36).

Two methods are commonly used to transfect baculovirus DNAs and plasmids into insect cells. Methods based on precipitation of DNA with calcium phosphate have been widely used, because the reagents are inexpensive and generally yield consistent results. Cationic lipids, such as Lipofectin™ (GIBCO/BRL), are becoming

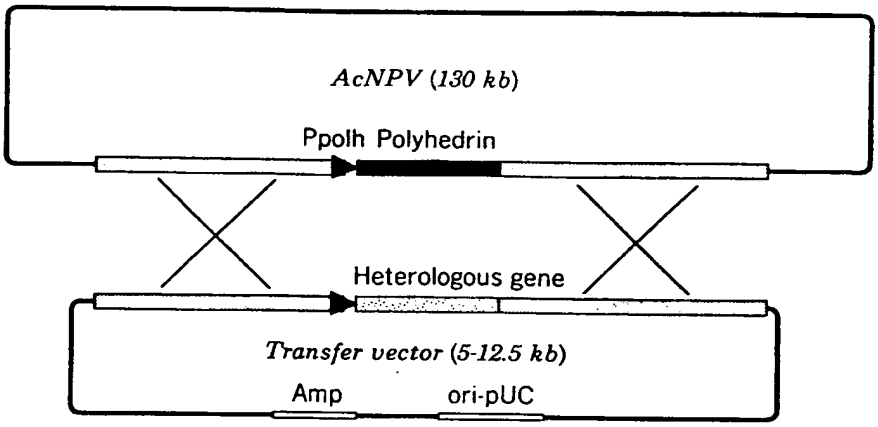


**Figure 7.4.** Generation of recombinant baculoviruses using circular AcNPV DNA. Baculovirus transfer vectors containing a heterologous gene under the control of the polyhedrin promoter are co-transfected into insect cells with circular genomic AcNPV DNA. The transfer vectors undergo homologous recombination with the parental virus to generate occlusion minus recombinant viral progeny that occur at a frequency of 0.1% to 1%.

more popular, however, as reports suggest that transfection efficiencies are 10-fold more efficient when using this reagent (74).

Plaque assays are used to facilitate the purification of recombinant viruses from transfection mixtures that contain nonrecombinant parent viruses and to determine the titer of virus stocks (in plaque-forming units/milliliter). Serial dilutions of virus are typically incubated with uninfected cells seeded at a low cell density for about an hour and then replaced with a semisolid overlay of media that contains agarose. After 5 days, plaques can be distinguished by the sparse density, accentuated by cell lysis, and larger size of infected cells compared to the dense background of uninfected cells. Recombinant baculoviruses that have insertions in the polyhedrin gene do not produce occluded virus within the nucleus of infected cells and can be distinguished from uninfected cells and wild-type AcNPV-infected cells by examining the plates under a stereo dissecting microscope.

Several viral stains have been used to enhance the visualization of plaques against the background of uninfected cells in a plaque assay. The most useful stain is MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is used to increase the contrast between the dead cells in a plaque and uninfected cells (75). The dye is usually added to the agarose overlay which slowly diffuses in, and is metabolized by, living cells to form a purple precipitate. Plaques appear yellow or clear against a deep purple background. This stain does not help distinguish plaques produced by occluded viruses from those produced by nonoccluded viruses.

The construction of recombinant baculoviruses by traditional transfection and plaque assay techniques can take as long as 4 to 6 weeks, and a wide variety of methods have been tried to accelerate this process (reviewed in references 34, 35, and 37). Some of these include limiting dilutions, plaque lifts, and cell affinity techniques. Methods which rely on detection of the expressed product by reaction with chromogenic substrates or by binding to antibodies have also been used to monitor the purification of recombinant viruses from mixtures containing non-

recombinant parent viruses. Methods that have been used to confirm the recombination event include not only plaque phenotype, but also analysis of the viral genome by DNA dot-blot hybridization, Southern blotting, and amplification of specific viral fragments by polymerase chain reaction.

## 7.7 SUMMARY OF POPULAR BACULOVIRUS TRANSFER VECTORS

A wide variety of baculovirus transfer vectors have been developed and used for constructing recombinant viruses (reviewed in references 34–37, 58, and 76). These can be grouped into two major classes: (i) vectors which are used to express a single heterologous gene and (ii) multiple-expression vectors which are designed to accept and express two or more heterologous genes. Each major class can be subdivided on the basis of the genomic locus used to target the insertion of the heterologous gene, the type of viral promoter used to drive expression of the heterologous gene, and the absence or presence of an ATG start codon to permit the expression of fusion proteins. The key features of several popular transfer vectors are described below.

The most commonly used baculovirus transfer vectors contain multiple cloning sites downstream from a polyhedrin promoter ($P_{polh}$). A wide variety of vectors have been constructed over the years that differ primarily in the length of the untranslated leader sequence and the position and selection of convenient cloning sites. Several versatile transfer vectors are now commercially available. The plasmid pBacIII (Invitrogen) contains 11 unique cloning sites in a synthetic polylinker region downstream from the polyhedrin promoter. The plasmids pBacPAK8 and pBacPAK9 (Clontech), which contain 18 unique cloning sites, differ in the orientation of the central portion of a synthetic polylinker. A variety of transfer vectors are also available that contain the *E. coli lacZ* gene under the control of a second promoter inserted upstream from the polyhedrin promoter (77–80). Recombinant viruses derived from these co-expression vectors produce blue plaques in the presence of X-gal, a chromogenic substrate for β-galactosidase. If a weak promoter is used to drive transcription of the *lacZ* gene, the β-galactosidase that is expressed does not usually interfere with the production or purification of the desired heterologous protein.

Several new vectors have been developed to facilitate the expression and affinity purification of fusion proteins. The pBlueBacHIS series of vectors (Invitrogen) can be used to express fusion proteins that contain a poly-histidine region at their amino terminus. The fusion proteins selectively bind to nickel-charged Sepharose resins and can be eluted under native or denaturing conditions. If nonfused proteins are desired, the metal binding domain of these poly-histidine fusions can be removed by treatment with enterokinase. Similar vectors have also been constructed that permit the expression of recombinant proteins as fusions to glutathione-*S*-transferase (GST) (81). The GST-fusion proteins are purified using glutathione affinity agarose, and the GST binding domain can be removed by treatment with thrombin.

Several baculovirus transfer vectors have been constructed that replace the polyhedrin promoter with weaker viral promoters that are active in earlier stages of infection. One such plasmid is pAcMP1, which contains the AcNPV basic protein promoter. Expression of β-galactosidase under the control of this promoter occurs between 8 and 24 hpi, with maximal synthesis between 12 and 15 hpi (82). Secretion of human chorionic gonadotropin (hCG) expressed under the control of this

promoter was higher than when the hCG gene was expressed under the control of the polyhedrin promoter (83). The specific activity of hCG expressed under the control of this earlier promoter was also higher than material expressed under the control of the very late polyhedrin promoter. These results are consistent with previous observations suggesting that some processing events, such as secretion and glycosylation, are compromised late in infection (56,84).

Multiple-expression vectors permit the simultaneous expression of two or more heterologous genes by a single recombinant virus. In pAcUW3 (85) and its derivatives pAcUW31 (Clontech) and pAcUW51 (Pharmingen), one gene is expressed under the control of the polyhedrin promoter, while the other is expressed under the control of the *p10* promoter. These plasmids have the *p10* promoter and SV40 poly(A) transcription termination sequences inserted upstream and directed in the opposite orientation from the polyhedrin promoter. One to two unique cloning sites are located downstream from each of the promoters in these plasmids. A more versatile plasmid is p2Bac (Invitrogen), which has the *p10* promoter and bovine somatotropin poly(A) signals inserted upstream from the polyhedrin promoter. The polylinker located downstream from the polyhedrin promoter has seven unique restriction sites, and the other polylinker downstream from the *p10* promoter has 19 unique restriction sites. Triple- and quadruple-expression vectors have been developed and used to facilitate the simultaneous co-expression of three or four bluetongue virus (BTV) structural proteins in baculovirus-infected insect cells (86). Plasmid pAcAB3 contains two *p10* promoters inserted on either side of a single polyhedrin promoter but oriented in opposite directions, and plasmid pAcAB4 contains two *p10* and two polyhedrin promoters. Double-capsid, virus-like particles consisting of the BTV proteins VP2, VP3, VP5, and VP7 are assembled in cells infected with a recombinant baculovirus derived from pAcAB4.

A variety of other transfer vectors which target the insertion of heterologous genes to other positions in the AcNPV genome have been constructed (reviewed in references 35, and 37). Most of these are vectors which direct the insertion of genes to the *p10* locus. The recombinant viruses generated with these vectors are often used in specialized applications, such as production of occluded recombinant viruses that are used to infect insect larvae by ingestion.

## 7.8 SUMMARY OF MODIFIED PARENT VIRUSES

Derivatives of AcNPV that are linearized at one or more locations facilitate the construction of recombinant baculoviruses by increasing the frequency of recombinant progeny present in a transfection mixture (reviewed in references 35–37). The proportion of recombinant viruses approaches 30% when a single unique site is introduced into the AcNPV genome near the polyhedrin locus (87–89). The total yield of infectious progeny, however, is reduced 15- to 150-fold. Recombination frequencies ranging from 86% to 99% are obtained when an improved linearized virus (marketed as BacPAK6 by Clontech and as BaculoGold by Pharmingen) is used as a parent virus (90). This virus contains three *Bsu*36I sites near the polyhedrin locus (Figure 7.5). One is in a *lacZ* gene under the control of the polyhedrin promoter, the second is in a nonessential gene upstream from the polyhedrin promoter, and the third is in an essential gene (ORF1629) downstream from the polyhedrin gene. The product of ORF1629 is a 78-kD protein that is either a virion envelope protein or a protein that occurs in the virion intermediate layer between the envelope

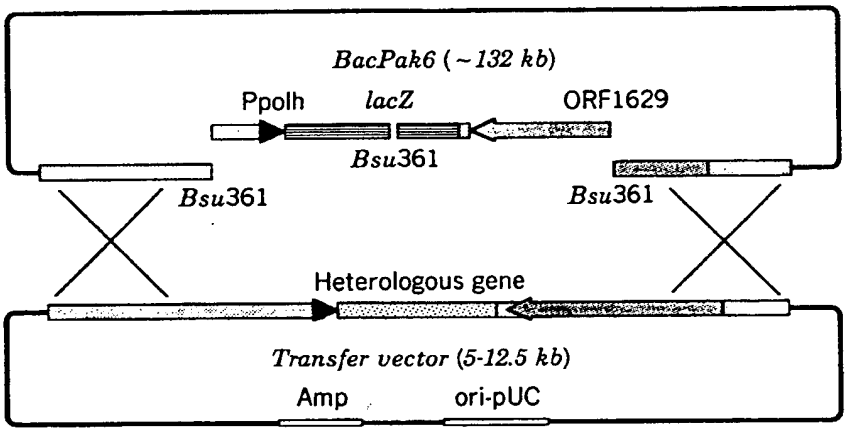


**Figure 7.5.** Generation of recombinant baculoviruses using BacPak6 parental virus DNA (90). BacPak6 parental virus DNA is linearized at three locations near the polyhedrin locus with *Bsu*36I. Homologous recombination between the parental virus and the transfer vector promotes circularization and restores ORF1629, which is an essential viral gene (92).

and nucleocapsid (91,92). The majority of the viable progeny that are produced have ORF1629 restored by homologous recombination between the transfer vector and the large *Bsu*36I fragment that contains the remainder of the viral genome. A variety of other modified viruses containing the *lacZ* gene inserted at other positions in the AcNPV genome may be useful as parent viruses for specialized applications (reviewed in references 35–37).

Two different approaches have been recently described that rely on the use of selectable or counterselectable markers to facilitate the construction of recombinant baculoviruses. In one study, the bacterial neomycin resistance gene (*neo*) and the AcNPV apoptosis suppressor gene (*p35*) were shown to function as dominant selectable markers when inserted into the AcNPV genome (93). When a transfer vector containing one of these markers is transfected into insect cells along with a parent virus that lacks the selectable marker, the proportion of the desired recombinants is enriched when selection for the marker gene is maintained. A different approach uses ganciclovir to prevent the replication of a parent virus that expresses the herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) gene under the control of the AcNPV IE1(0) immediate-early promoter (94). When this parent virus is transfected into cells with a baculovirus transfer vector lacking the HSV-*tk* cassette and grown in the presence of ganciclovir, over 85% of the viral progeny are recombinant viruses. Replication of wild-type AcNPV and recombinant viruses lacking the HSV-*tk* cassette is not inhibited by the 100 μM ganciclovir used to prevent replication of the HSV-*tk* containing parent virus.

## 7.9 NOVEL STRATEGIES TO GENERATE RECOMBINANT BACULOVIRUSES

A variety of novel strategies to generate recombinant baculoviruses have been described that do not rely on homologous recombination between a transfer vector and a parent virus in cultured insect cells. This recombination is usually the least efficient step in the generation of a recombinant virus, and identifying and purifying

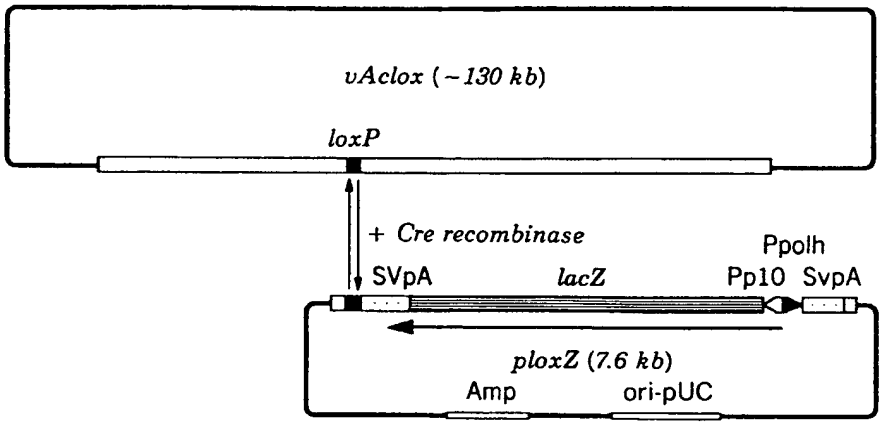


**Figure 7.6** Generation of recombinant baculoviruses using the Cre/*lox* system (95). The transfer vectors and parent virus DNAs contain synthetic *lox* sites that recombine *in vitro* in the presence of the Cre enzyme of bacteriophage P1. Single insertions of the transfer vector into the parent virus represent about 50% of the viral progeny. The remainder represent no insertions or multiple insertions of the vectors into each other.

recombinant baculoviruses from a background of nonrecombinant parent viruses by traditional plaque assay methods can often take more than a month. Variations on the following approaches, with modest improvements, may supplant many of the traditional vectors and methods used to generate recombinant baculoviruses in the years ahead.

One method to facilitate the construction of recombinant baculoviruses relies on site-specific recombination between a transfer vector and a parent virus *in vitro* (95). Both the transfer vector and the parent virus were modified to contain a short segment (*lox*) that is recognized by the Cre enzyme of bacteriophage P1 (Figure 7.6). When these DNAs are incubated in the presence of purified Cre enzyme, they recombine at the inserted *lox* sites. High recombination frequencies are possible, but no more than 50% of the viral progeny have the desired recombinant phenotype.

A second method to facilitate the construction of recombinant baculoviruses is based on the use of a baculovirus shuttle vector that can propagate in yeast (96). The shuttle vector contains ARS and CEN sequences, which ensure stable replication in yeast, and two selectable marker genes (URA3 and SUP4-o) inserted as a cassette into the AcNPV genome downstream from the polyhedrin promoter (Figure 7.7). Recombinant shuttle vectors are constructed by homologous recombination in yeast between the shuttle vector and a transfer vector that contains a yeast selectable marker. Colonies containing the recombinant shuttle vectors are identified by screening for a color change and resistance to canavanine, a toxic amino acid analogue. The selection is inefficient, however, because of the low frequency of transformation and a high background of reversion to resistance by the host strain. Recombinant shuttle vector DNA can be purified from total yeast DNA over sucrose gradients and used to transfect insect cells. Stocks of recombinant viruses can be generated within 10 to 12 days, bypassing the need for purification by multiple plaque assays. The need for expertise in yeast genetics, low transformation efficiencies, and tedious DNA purification methods are limitations of this approach and may prevent widespread use of this system.

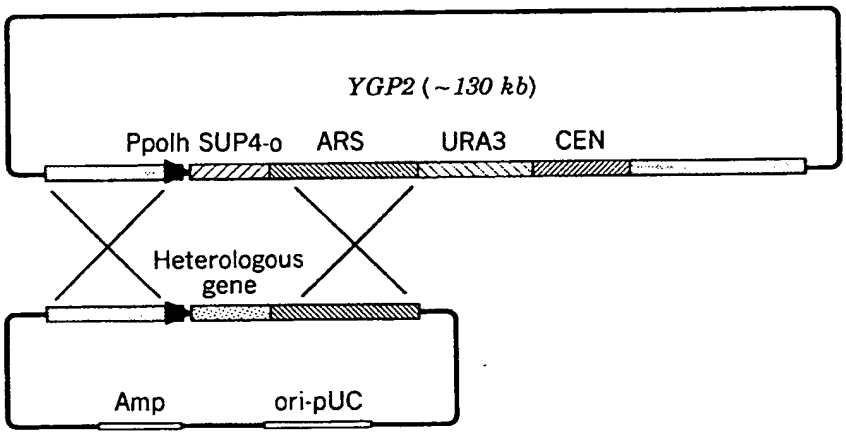


**Figure 7.7.** Generation of recombinant baculoviruses using a baculovirus shuttle vector that can propagate in yeast (96). A baculovirus shuttle vector that contains replication elements (ARS and CEN) that function in yeast can undergo homologous recombination with a modified transfer vector that contains an ARS element downstream from the heterologous gene. Yeast colonies containing recombinant shuttle vectors are identified by a color change on agar plates and confer resistance to a toxic amino acid analogue. Recombinant shuttle vector DNA is infectious when introduced into insect cells where it expresses the heterologous gene under the control of the polyhedrin promoter.

Recently, Luckow et al. (97) described a rapid and efficient method to generate recombinant baculoviruses that is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli* (Figure 7.8). The bacmid contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lacZ*α peptide from a pUC-based cloning vector. Inserted into the N-terminus of the *lacZ*α gene is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-*att*Tn7) that does not disrupt the reading frame of the *lacZ*α peptide. The shuttle vector propagates in *Escherichia coli* DH10B as a large plasmid that confers resistance to kanaymcin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue ($Lac^+$) in the presence of X-gal and IPTG. Composite shuttle vectors are constructed by transposing a mini-Tn7 from a donor plasmid to the mini-*att*Tn7 on the parent shuttle vector when Tn7 transposition functions are provided in *trans* by a helper plasmid. The mini-Tn7 contains a gentamicin resistance marker, a baculovirus promoter, a heterologous gene, and an SV40 poly(A) signal inserted between the left and right arms of Tn7. Transposition of the mini-Tn7 into the mini-*att*Tn7 on the parent bacmid disrupts expression of the *lacZ*α peptide, so that colonies harboring the composite shuttle vector are white instead of blue. Composite bacmid DNAs can be isolated by purification over resin columns and used to transfect insect cells. Heterologous genes inserted into the composite viruses are expressed at levels that are similar to those observed by viruses constructed by traditional methods. Because the composite bacmid DNA is not contaminated with parental bacmid DNA, plaque assays are eliminated, and pure stocks of recombinant (composite) viruses can be generated within 7 to 10 days. The only significant disadvantage of this approach is that existing baculovirus transfer vectors cannot be used because the heterologous gene must be cloned into a Tn7-based donor plasmid. Donor plasmids are small, however, and easily manipulated to